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RESEARCH ARTICLE

The first record of the trematode *Urogonimus certhiae* (Trematoda: Leucochloridiidae) in the Eurasian nuthatch *Sitta europaea*

Первая находка трематоды *Urogonimus certhiae* (Trematoda: Leucochloridiidae) у обыкновенного поползня *Sitta europaea*

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Abstract. Adult trematodes of the genus *Urogonimus* Monticelli, 1888 were found in the cloaca of a male Eurasian nuthatch *Sitta europaea* Linnaeus, 1758, the carcass of which was found in the Vyritsa Settlm. (Leningrad Prov., Northwest Russia). Based on morphological characters, the worms were identified as *U. certhiae* McIntosh, 1927. This is the first record of this parasite from the nuthatch and from Northwest Russia. We analysed nucleotide sequences of ITS1-5.8S-ITS2 rDNA of this species and found important differences with *U. macrostomus* (Rudolphi, 1803). Genetic and morphological data indicated that *U. certhiae* and *U. macrostomus* were two separate species. Phylogenetic analysis confirmed that *Urogonimus* and *Leucochloridium* Carus, 1835 were two distinct genera.

Резюме. В поселке Вырица Ленинградской области был обнаружен труп самца обыкновенного поползня *Sitta europaea* Linnaeus, 1758, в клоаке которого были найдены мариты рода *Urogonimus* Monticelli, 1888. На основании морфологических признаков черви были определены как *U. certhiae* McIntosh, 1927. Это первое обнаружение данного вида паразитов в поползне и в Северо-Западном регионе России. Выполнен анализ нуклеотидных последовательностей ITS1-5.8S-ITS2 рДНК *U. certhiae* и установлены их отличия от последовательностей этих генов у *U. macrostomus* (Rudolphi, 1803). Генетические и морфологические данные позволяют считать эти виды самостоятельными. Филогенетический анализ подтвердил различие родов *Urogonimus* и *Leucochloridium* Carus, 1835.

Key words: trematodes, genotyping, rDNA, Leucochloridiidae, *Urogonimus certhiae, Sitta europaea*

Ключевые слова: трематоды, генотипирование, рДНК, Leucochloridiidae, *Urogonimus certhiae, Sitta europaea*

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Introduction

In the ten years of our studies of trematodes from the snail *Succinea putris* Linnaeus, 1758 in the Leningrad Prov. (Northwest Russia), we have found three trematode species: *Leucochloridium paradoxum* Carus, 1835, *L. perturbatum* Pojmanska, 1969 and *L. vogtianum* Baudon, 1881 (Ataev et al., 2016; Prokhorova et al., 2017). No other species from the family Leucochloridiidae have ever been recorded in this region or neighbouring regions of Russia. A fresh carcass of the nuthatch (*Sitta europaea* Linnaeus, 1758) was found in the Vyritsa Settlem. (Leningrad Prov., Russia) in 2016. Dissection showed that it harboured trematode adults. Their localisation in the cloaca and general structure suggested that they belonged to the family Leucochloridiidae. We identified the species as *Urogonimus certhiae* McIntosh, 1927 using morphological and molecular genetic characters and chose fragments of the cluster of ribosomal genes, including ITS1-5.8S-ITS2 rDNA as a molecular marker.

Materials and methods

Morphological study

A carcass of a male nuthatch was found near Vyritsa Settlem. (Leningrad Prov., Russia, 59°24′40″N 30°20′50″E) in August 2016. After dissection, 74 adult flukes were found in the cloaca. All of them were fixed using a “cold fixation” technique (Bakke, 1978) in 70% ethyl alcohol for morphological and molecular genetic analysis. For preparations of whole mounts, the adults were stained with alum-carmine. They were drawn using a Leica DM 1000 microscope equipped with a drawing tube. A Leica DM 5000 microscope and Image Scope software was used for the morphometric analysis of 7 adults. The whole mounts of adults of *U. certhiae* were deposited at the Department of Zoology of Herzen State Pedagogical University, St Petersburg (collection no. 1.16.1–1.16.7).

DNA sequencing and phylogenetic analysis

Worms for molecular genetic analysis were stored at −80 °C. Chromosomal DNA was extracted from each worm (n = 13) individually by phenol–chloroform extraction from nuclei purified by centrifuging through a sucrose cushion (Sambrook & Russel, 2001).

For amplification of the 18S-28S rDNA fragment, we used four pairs of specific primers designed previously for the genus *Leucochloridium* Carus, 1835. PCRs were run in a Tercyc thermocycler (DNA-Technology, Russia) following the protocol of Zhukova et al. (2014). PCR products were analysed using a 1.4% agarose gel in TBE buffer and sequenced using an ABI PRISM 310 sequencer (Applied Biosystems). Assembly and multiple alignments of nucleotide sequences and analysis of the chromatograms were performed using BioEdit software (Hall, 1999). BLAST software at the NCBI server was used (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the homology of nucleotide sequences.

The folding of the sequences into putative secondary structures was performed with Mfold version 3.0 (Zuker, 2003).

Phylogenies were reconstructed based on ITS1-5.8S-ITS2 nucleotide sequences. Outgroup and ingroup representatives are given in Table 1. An optimal mathematical model for calculating genetic distances was chosen using the Akaike information criterion (AIC) with assistance from jModelTest 2.1.7 software (Darriba et al., 2012). The best-fitting model for ITS1-5.8S-ITS2 partition was the Kimura two-parameter model with the use of gamma correction (Kimura, 1980). Bootstrap branch support (BS) levels were estimated with 1000 replicates. MEGA X software was used for maximum-likelihood phylogenetic reconstructions (Kumar et al., 2018).

The primary sequence of rRNA was transformed into a putative secondary structure at a folding temperature of 44 °C, the average physiological temperature in the cloaca of passerine birds. Secondary structures had the highest negative free energy (dG).

Results

Adult morphology

Immediately after dissection of the nuthatch, it became clear from the localisation and morphology of the adults found in its cloaca that they belonged to the family Leucochloridiidae. Their

Table 1. Nucleotide sequences used for the phylogenetic analysis of Leucochloridiidae trematodes.

Family	Species	GenBank accession number
Leucochloridiidae	*Leucochloridium perturbatum*	KP938186
	Leucochloridium paradoxum	KP938187
	Leucochloridium vogtianum	KU351661
	Urotocus rossitensis	KP903635
	Urogonimus macrostomus	KP903705
	Urogonimus certhiae	MK347524 (this study)
Leucochloridiomorphidae	*Leucochloridiomorpha constantiae*	MK411399
	Leucochloridiomorpha lutea	MK411398
Echinostomatidae	*Echinostoma caproni*	U58098
	Echinostoma paraensei	U58100
	Echinostoma trivolvis	AF067852
Fasciolidae	*Fasciola hepatica*	MG569975
	Fasciola gigantica	KF543340
Schistosomatidae	*Schistosoma japonicum*	FJ852563
	Schistosoma mansoni	AF531314
	Schistosoma rodhaini	AF531312

bodies were oval, with a slightly pointed posterior end (Fig. 1, Table 2). There were numerous spines on the body surface. The oral sucker was larger than the ventral one. The oesophagus was absent. The pharynx extended further than the posterior end of the oral sucker. The caeca started from the pharynx, turned a little towards the anterior body end (to the level of the pharynx) and then passed laterally towards the posterior body end, terminating at the level of the posterior side of the lower testis. Vitellaria were largely extracaecal, starting at the level of the posterior third of oral sucker to approximately the posterior third of the ovary, not reaching caecal extremities (Fig. 1).

The space behind the ventral sucker was mostly filled with gonads, which were arranged in a straight line. Testes were larger than ovaries. The uterus ascended in loops on the left side to the midlevel of the oral sucker, then reaching that level on the right by passing around the posterior margin of the acetabulum, and descending to the genital pore at or slightly dorsal to the posterior end. The genital pore was located terminally. The unarmed cirrus-sac was rounded and smaller than the ovary.

Based on the morphological analysis, we attributed the worms to the species *Urogonimus certhiae* (see below for its differences from congeneric species).

Molecular identification

We obtained a 1758 base pair fragment of the rDNA (MK347524). It included complete nucleotide sequences of the ITS1, ITS2 and 5.8S and partial sequences of the18S and 28S rDNA. The sequenced DNA fragment was identical in all the adults (n = 13), indicating that they belonged to the same species.

To identify the species, we used full nucleotide sequences of ITS1-5.8S-TS2 fragments. Representatives of the families Leucochloridiomorphidae, Echinostomatidae, Shistosomatidae, Fasciolidae and other species of the families Leu-

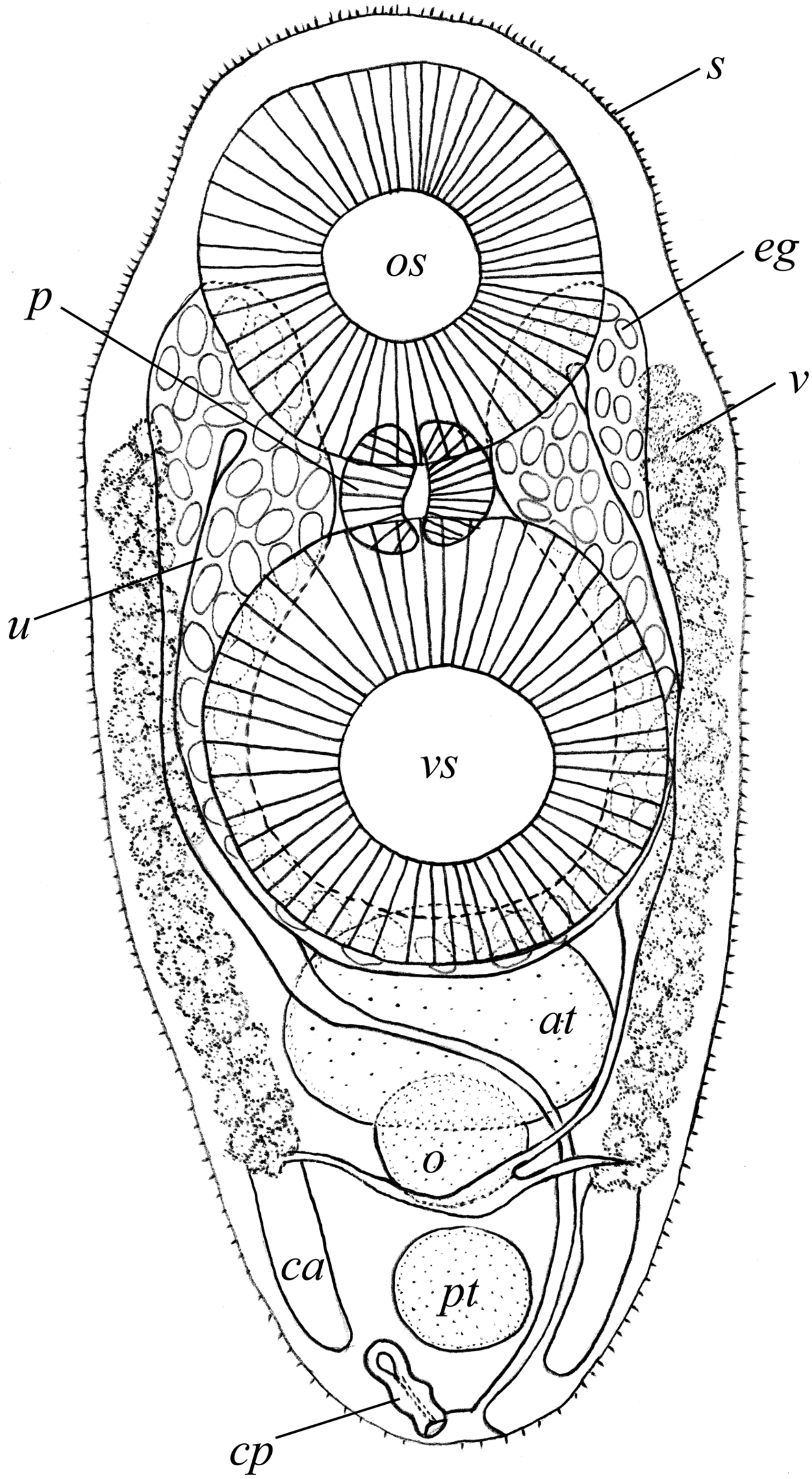


Fig. 1. Adult trematode *Urogonimus certhiae* isolated from a *Sitta europaea* specimen. *at*, anterior testes; *ca*, caeca; *cp*, cirrus pouch; *eg*, egg; *o*, ovary; *os*, oral sucker; *p*, pharynx; *pt*, posterior testes; *s*, spines; *u*, uterus; *v*, vitellaria; *vs*, ventral sucker. Scale bar: 160 μm.

Table 2. Morphometric data on *Urogonimus certhiae* and *U. macrostomus*. Data are given in micrometers as a range and mean ± standard deviation.

	U. certhiae from *Sitta europaea* (this study)	*U. certhiae* from *Certhia americana* Bonaparte, 1838 (after McIntosh, 1927)	*U. macrostomus* from *Sturnus vulgaris* Linnaeus, 1758 (after Bakke, 1978)*
Number of specimens	7	1	20
Body	988–1460 × 440–641 (1146 ± 138 × 509 ± 68)	1684 × 1020	1590–2560 × 610–1090 (1900 ± 150 × 800 ± 70)
Oral sucker	240–395 × 297–476 (336 ± 46 × 344 ± 59)	453 × 534	390–570 × 375–585 (451 ± 24 × 499 ± 44)
Ventral sucker	242–420 × 269–411 (303 ± 58 × 320 ± 44)	486 × 534	375–600 × 375–615 (444 ± 33 × 449 ± 35)
Pharynx	110–177 × 106–153 (125 ± 24 × 124 ± 17)	152 × 224	150–218 × 150–248 (173 ± 10 × 185 ± 14)
Anterior testes	134–171 × 123–222 (156 ± 16 × 156 ± 47)	226 × 256	150–308 × 128–225 (212 ± 19 × 174 ± 11)
Posterior testes	97–216 × 135–202 (159 ± 55 × 160 ± 25)	226 × 240	150–270 × 135–188 (193 ± 13 × 165 ± 9)
Ovary	83–124 × 127–170 (103 ± 15 × 138 ± 19)	120 × 200	113–180 × 98–158 (148 ± 8 × 130 ± 8)

* “Cold fixation” in 70% ethyl alcohol. We used the same fixation protocol to make the data comparable.

cochloridiidae, whose nucleotide sequences are present in GenBank, were used for phylogenetic reconstruction. The closest genotyped species on the obtained tree was *Urogonimus macrostomus* (Rudolphi, 1803). Our sample diverged from the common node with *U. macrostomus*, indicating a reliable genetic differentiation between these species (Fig. 2).

Based on the sequenced rDNA locus, the homology between the studied species, identified as *U. certhiae* based on morphological characters, and *U. macrostomus* was 96.24%. The divergences, including single nucleotide substitutions and two-nucleotide insertions, were present in the ITS2 region (homology 94.45%) and the 5.8S genes (homology 94.51%) and 28S genes (homology 95.78% at the sequenced fragment). Homology of the ITS1 region was 99.76%.

The nucleotide sequences ITS2 were used to obtain topological schemes of the secondary structures of their transcripts (Fig. 3), allowing one to visualise differences between *U. certhiae* and *U. macrostomus*. Graphic images of the secondary structures of the ITS2 transcripts in *U. certhiae* and *U. macrostomus* revealed a similar topology. They are characterised by seven hairpins, the fifth hairpin being the largest. At the same time, *U. certhiae* has an additional loop on the third hairpin, and that of *U. macrostomus,* on the fourth hairpin. In addition, the first hairpin of the *U. certhiae* transcript is longer than that of *U. macrostomus*.

Diagnosis

The adults found in the nuthatch were attributed to the genus *Urogonimus* Monticelli, 1888 based on the following morphological characters: genital pore located subterminally, small cirrus pouch, uterus forming two ascending and two descending loops crossing the body horizontally in the posterior part of the ventral sucker and gonads arranged in a straight line.

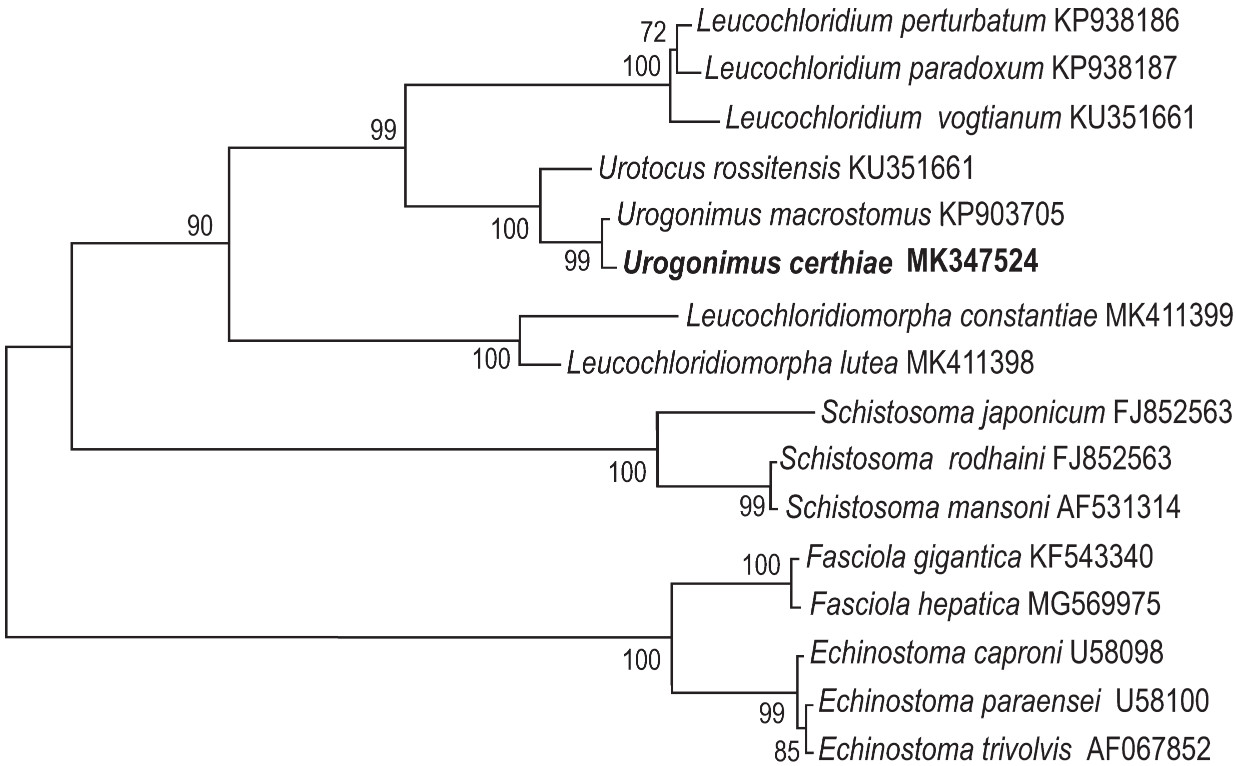


Fig. 2. Maximum likelihood phylogenetic tree based on the ITS1-5.8S-ITS2 sequences of trematodes of the family Leucochloridiidae. Outgroups: Fasciolidae, Schistosomatidae, and Echinostomatidae. The topology was inferred by a Kimura two parameter model with gamma correction. The numbers below the branches are bootstrap values (1000 replicates). The nucleotide sequence obtained in this study is in bold.

Differential diagnosis

Based on the structure of adult worms, *U. certhiae* are most similar to *U. macrostomus*. The main morphological distinctions of *U. certhiae* are spines on the tegument present *vs* absent in *U. macrostomus,* and loops of the uterus reach as far as the posterior part of the oral sucker *vs* loops of the uterus do not reach oral sucker in *U. macrostomus*. *Urogonimus certhiae* differs from *U. macrostomus* in smaller size – mean body length 1146 μm *vs* 1900 μm in *U. macrostomus*, mean body width 509 μm *vs* 780 μm in *U. macrostomus*; smaller gonads – mean ovary length 103 μm in *U. certhiae vs* 148 μm in *U. macrostomus*, mean anterior testes length 156 μm in *U. certhiae vs* 212 μm in *U. macrostomus* and mean posterior testes length 159 μm in *U. certhiae vs* 193 μm in *U. macrostomus*.

The differences in ITS2 and 5.8S fragments between *U. certhiae* and *U. macrostomus* are, respectively, 6.5% and 6.1%. Interspecific differences between *U. certhiae* and *U. macrostomus* also visualised well in the secondary structures of the ITS2 transcripts. *U. certhiae* has an additional loop on the third hairpin, while *U. macrostomus,* on the fourth hairpin. In addition, the first hairpin of *U. certhiae* transcript is longer than that of *U. macrostomus*.

Discussion

The worms obtained from the nuthatch could be fairly reliably attributed to *U. certhiae* based on morphological features described by McIntosh (1927). This species has not previously been recorded from nuthatches, nor has it ever been found in Northwest Russia. Morphologically, the adults of *U. certhiae* are similar to *U. macrostomus*, and their distribution in Europe is also similar (Bakke, 1978). However, adult worms of *U. cer-*

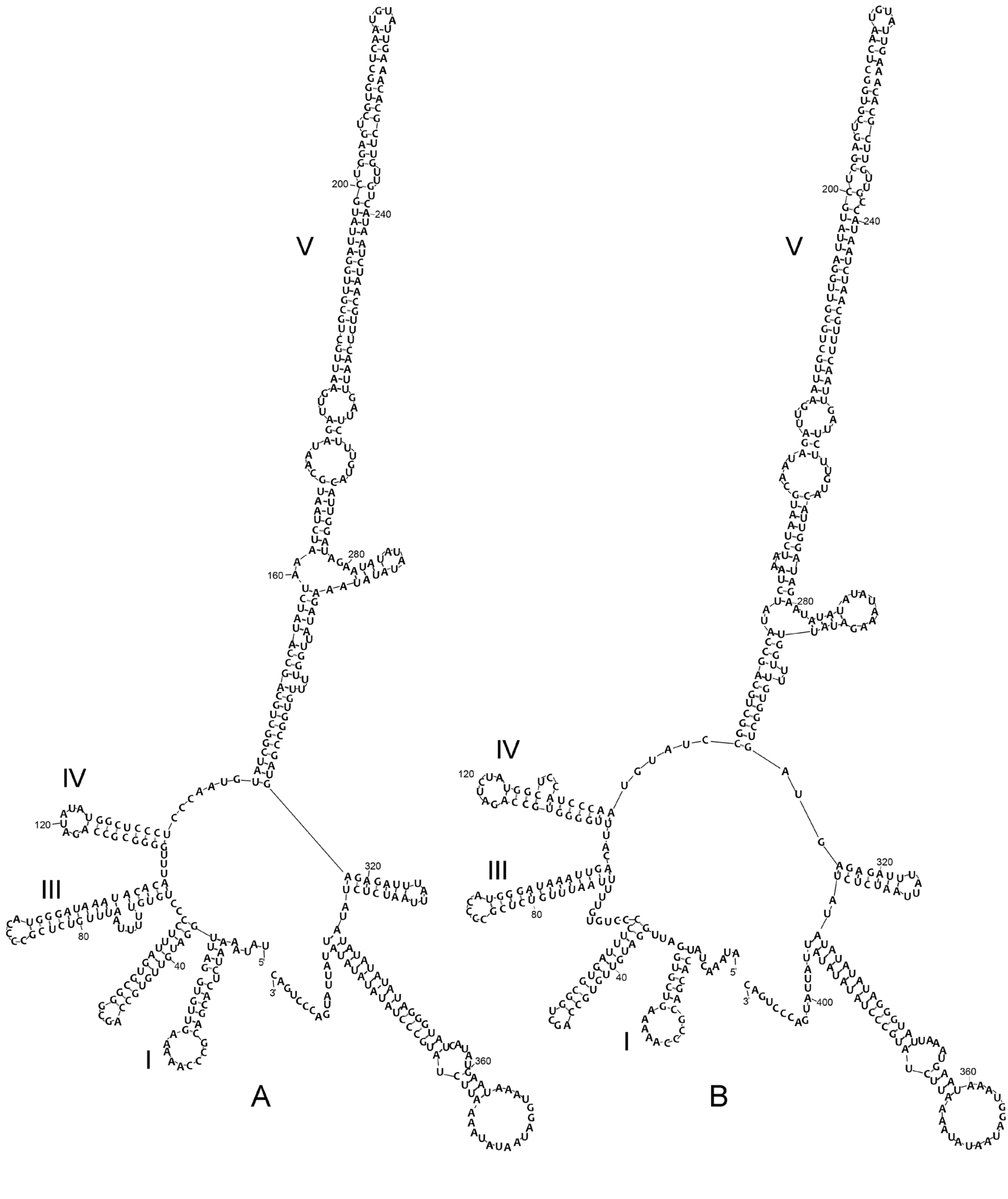


Fig. 3. Predicted secondary structures of transcripts of ITS2 of trematodes *Urogonimus* spp. **A**, *U. certhiae* (416 bp; dG = −100.69); **B**, *U. macrostomus* (410 bp; dG = −97.14). dG: predicted free energy in kcal/mol.

thiae bear numerous spines on their surface and have smaller ovaries (Table 2). Furthermore, the uterus of these trematodes passes into the area of the oral sucker (Fig. 1).

Morphological differences between these species were confirmed by molecular genetic analysis. The degree of genotyping differences in the ITS1-5.8S-ITS2 region corresponded to the interspecies differences within other genera of the family Leucochloridiidae (Ataev et al., 2016; Locke et al., 2012). For example, the differences in ITS2 and 5.8S fragments between *Urogonimus certhiae* and *U. macrostomus* amount, respectively, to 6.5% and 6.1%. Differences between *Leucochloridium paradoxum* and *L. perturbatum* in these genome fragments amount to 7.2% and 0.6% (Ataev et al., 2016). Thus, these sites of the genome are the most suitable markers for distinguishing species within genera of the family Leucochloridiidae.

We reconstructed the secondary structures of their transcripts to identify the importance of differences in the ITS2 nucleotide sequences. Differences in the secondary structure of ITS2 indicate that the samples belong to different species (Fig. 3). This structural difference can be used as an additional feature for species identification within the *Urogonimus* genus.

Thus, we performed the first complex species identification of the trematode *U. certhiae* using morphological and molecular genetic characters. Our data confirmed the taxonomic significance of the emended morphological diagnosis of this species. The nucleotide sequence of 18S-ITS1-5.8S-ITS2-28S fragments is unique for *U. certhiae* and may be used for species identification.

Our results confirm that *Leucochloridium* Carus, 1835 and *Urogonimus* Monticelli, 1888 are two independent genera. For a long time, some trematode species have been attributed alternatively to one or the other genus. Morphological characters of representatives of these two genera were clearly described by Kagan (1952). The main difference is that the adults of *Urogonimus* have two loops of the uterus. Its middle portion descends to the posterior edge of the ventral sucker. In species of *Leucochloridium*, the loops are less pronounced, and the middle portion of the uterus descends only to the anterior part of the ventral sucker. In the posterior body part of adult worms from the genus *Urogonimus*, the vitellaria do not reach the ends of the caeca, while in species of *Leucochloridium* the vitellaria almost reach the cirrus pouch. In contrast to that of *Leucochloridium,* the cirrus of *Urogonimus* is short and unarmed. Another important difference is the arrangement of the gonads: they form a triangle in *Leucochloridium* adults and are arranged in a straight line in *Urogonimus* adults (Fig. 1).

Our molecular genetic analysis proved that the separation of *Leucochloridium* and *Urogonimus* is justified. The phylogenetic reconstruction showed that the genus *Urogonimus* is grouped with the genus *Urotocus* Looss 1899 and they are both separate from the genus *Leucochloridium*, forming independent clades within the family Leucochloridiidae (Fig. 2).

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